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# Combined Mutation Screening of NKX2-5, GATA4, and TBX5 in Congenital Heart Disease: Multiple Heterozygosity and Novel Mutations

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## ABSTRACT

**Background.** Variants of several genes encoding transcription modulators, signal transduction, and structural proteins are known to cause Mendelian congenital heart disease (CHD). *NKX2-5* and *GATA4* were the first CHD-causing genes identified by linkage analysis in large affected families. Mutations of *TBX5* cause Holt–Oram syndrome, which includes CHD as a clinical feature. All three genes have a well-established role in cardiac development.

**Design.** In order to investigate the possible role of multiple mutations in CHD, a combined mutation screening was performed in *NKX2-5*, *GATA4*, and *TBX5* in the same patient cohort. Samples from a cohort of 331 CHD patients were analyzed by polymerase chain reaction, double high-performance liquid chromatography and sequencing in order to identify changes in the *NKX2-5*, *GATA4*, and *TBX5* genes.

**Results.** Two cases of multiple heterozygosity of putative disease-causing mutations were identified. One patient was found with a novel L122P *NKX2-5* mutation in combination with the private A1443D mutation of *MYH6*. A patient heterozygote for a D425N *GATA4* mutation carries also a private mutation of the *MYH6* gene (V700M).

**Conclusions.** In addition to reporting two novel mutations of *NKX2-5* in CHD, we describe families where multiple individual mutations seem to have an additive effect over the pathogenesis of CHD. Our findings highlight the usefulness of multiple gene mutational analysis of large CHD cohorts.

**Key Words.** Congenital Heart Disease; Mutations; Multiple Heterozygosity

## Introduction

Congenital heart disease (CHD) is a complex trait, as both environmental and genetic factors have been implicated in its pathogenesis. The etiology of CHD has a strong genetic component, as shown by extensive epidemiological studies in large series of consecutive births.<sup>1,2</sup> In approxi-

mately one in four cases, CHD occurs associated with other congenital anomalies within a single-gene disorder (e.g., Holt–Oram syndrome), sporadic malformative complex (e.g., VACTERL association), or as a consequence of a chromosomal abnormality (e.g., trisomy 21).<sup>3</sup> Cases of isolated CHD appear mainly as sporadic events. However, a small fraction present as familial cases, often showing Mendelian segregation with widely variable penetrance.<sup>4</sup> The first genes involved in Mendelian isolated CHD, *NKX2-5* and *GATA4*, which encode transcription factors, were identified

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by genetic linkage studies in large affected families.<sup>5,6</sup>

The mammalian *NKX2-5* gene was discovered during a screening for mouse homologues of *tinman*,<sup>7,8</sup> a *Drosophila* gene essential for cardiac development,<sup>9,10</sup> the product of which has been categorized as a Class I NK-2 homeodomain protein.<sup>11</sup> This group of transcription factors bind to the 5'-CAAGTG-3' motif in target promoters.<sup>12</sup> Murine *NKX2-5* is expressed from day 7 in the cardiac primordia<sup>8</sup> and is an early marker of both embryonic heart fields.<sup>13</sup> Although mutations of *NKX2-5* are associated with a wide spectrum of CHDs and thyroid dysgenesis,<sup>14</sup> most manifest as atrial septal defects (ASDs) and atrioventricular block.<sup>15</sup>

The product of *GATA4* belongs to the zinc-finger family of transcription factors and, as the other GATA proteins, it binds to the 5'-(A/C/T)GATA(A/G)-3' motif within its target sequences.<sup>16</sup> The mammalian *GATA4* gene was identified in a screen of zinc-finger encoding cDNAs in a mouse library,<sup>17</sup> and it was first suspected to be implicated in CHD when a deletion<sup>18</sup> and a duplication<sup>19</sup> of the chromosomal segment in which it is contained were discovered in patients with cardiac malformation. Most mutations of *GATA4* occur in the segments of the gene encoding the zinc-finger motifs<sup>16</sup> and have been related to diverse types of CHDs.<sup>20</sup>

Mutations of *TBX5* cause Holt–Oram syndrome, the most common of the heart–hand syndromes.<sup>21,22</sup> If strict criteria are used in Holt–Oram syndrome diagnosis (i.e., at least one family member with radial ray defect and cardiac septal defects), mutation of *TBX5* is found in more than 70% of cases.<sup>23</sup> The vertebrate *TBX5* gene was discovered in a screen of mouse cDNA clones using a probe complementary to the *TBX2* T-box region.<sup>24</sup> It encodes a transcription factor and alternative splicing regulator<sup>25</sup> expressed in the developing heart, among other tissues.<sup>21</sup> Except for somatic mutations,<sup>26</sup> no variants of *TBX5* have been found to cause nonsyndromic CHD.

Physical and functional interaction to modulate transcription of cardiac genes has been documented between the *NKX2-5*, *GATA4*, and *TBX5* proteins.<sup>5,27,28</sup> In order to evaluate the contribution to multiple mutations in these genes in the pathogenesis of CHD, we conducted a mutational scan of the *NKX2-5*, *GATA4*, and *TBX5* genes in a large cohort of sporadic nonsyndromic CHD cases that complements the findings of our previous analysis of *MYH6* in the same cohort.<sup>29</sup> We found two novel

mutations of the *NKX2-5* gene and two instances in which the distribution of changes in families is consistent with additive effects of variants in different genes in the pathogenesis of CHD.

## Methods

### Patients and Samples

The patient cohort comprised 331 patients with a wide variety of CHDs. Peripheral blood samples from all participants were taken after informed consent and approval of the project by the local ethics committees. Genomic DNA was purified from blood using the QIAmp DNA blood Maxi kit (Qiagen, Hilden, Germany) following the manufacturer directions. Anonymous human control DNA panels were obtained from the European Collection of Cell Culture (Salisbury, UK).

### Denaturing High Performance Liquid Chromatography (dHPLC)

Mutational analysis by dHPLC was performed as described previously.<sup>30</sup> Briefly, to analyze the combined 18 exons of the *NKX2-5*, *GATA4*, and *TBX5* genes, 35 polymerase chain reaction (PCR) amplicons were designed. Most of the amplicons spanned individual exons and short segments of flanking introns to each side to detect mutations of splicing regulatory elements. Large exons were spanned by two overlapping amplicons. A pair of PCR primers was designed for each amplicon (see Table 1). PCR reactions were performed using patient and control DNA samples following standard protocols. A final hybridization step for heteroduplex formation was carried out heating the PCR products to 95°C and cooling down 1.5°C per minute until a temperature of 25°C was reached. Sequences of individual amplicons were processed by the Navigator software (Transgenomic, Omaha, NE, USA) in order to plot the melting profile of each DNA segment to determine the optimal dHPLC temperatures. PCR products were analyzed on the dHPLC WAVE System (Transgenomic). PCR products displaying a trace indicative of heterozygosity were sequenced by standard protocols. Novel potentially deleterious variants were screened by dHPLC in samples from 384 ethnically matched control subjects.

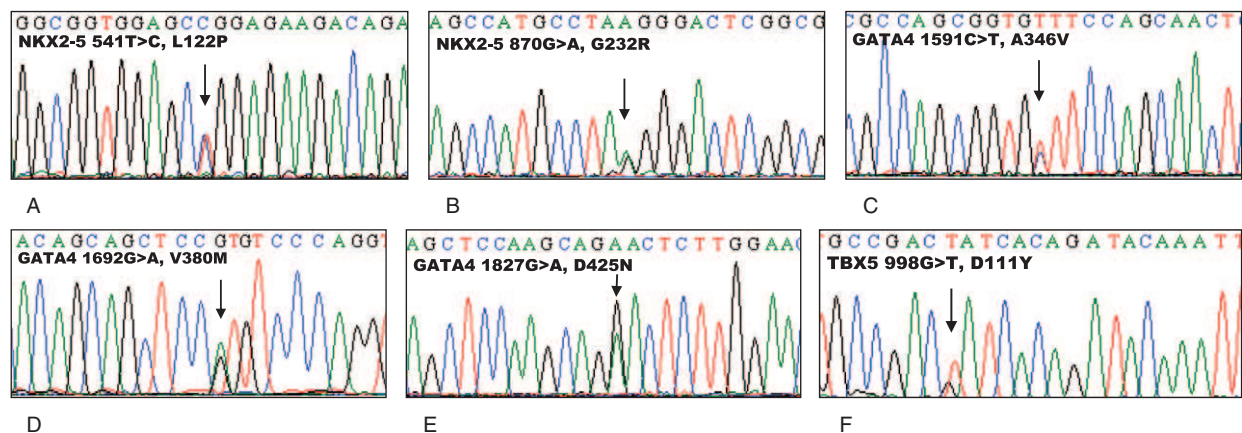
## Results

We identified two novel nonsynonymous mutations in *NKX2-5* compared with sequence accession number NM\_004387.2 for the cDNA and

**Table 1.** Summary of the Primers, Size, Annealing Temperature (Ta) of the Amplicons Used for the PCR Amplification of the *NKX2-5*, *GATA4*, and *TBX5* Genes and Temperatures Used in dHPLC

Amplicon	Forward Primer	Reverse Primer	Size (bp)	Ta	dHPLC Temperatures
NKX2.5-E1P1	tgacacgaaactgctcatcg	gtaggcctctggcttgaagg	416	56.6	63.3, 65.3, 67.3
NKX2.5-E1P2	ctggcgctgtgagactgg	agtttctggggacgaaagc	422	56	62, 63.6, 66.2, 67.4
NKX2.5-E2P1	caagccgctcttaccgaagc	cgttataaccgtaggattgagg	467	59.6	62.4, 65.6, 66.4
NKX2.5-E2P2	ccatgctaggggactcgc	gggggacagctaagacacc	530	60.9	62.2, 63.9
NKX2.5-E2P3	attcactcctgcggagacc	tcaatttgcctcaggaatgc	461	54.1	59.3, 62.8, 64, 66.3
GATA4-E1	gtagcactgggcatcttcc	ctacctccagacaagcaaaag	389	58.4	62.1, 64.3, 67.7
GATA4-E2P1	gtgggttctgaaagctctgg	cctcgtgtcctctctctcc	497	58.4	57.4, 59.6, 62.3, 65.1
GATA4-E2P2	cacgcatattatcgtgttgc	gccttgaggtaggacagc	267	54.7	64.3, 66.1, 68.8
GATA4-E2P3	cgctcctgccagctacg	gtcccgggaaggagaag	586	60.9	66.2, 68.2, 68.8
GATA4-E3	aaagggcattgttctgtgc	agaggatgtcccacaagc	344	54.1	58.6, 61.5, 64.1, 64.7
GATA4-E4	gagttaggtgccgtcacagg	gagagatgggcatcagaag	336	60.3	63.2, 65.1
GATA4-E5	cagggtgtgtcttcaatgc	tgattcttaggcactctgagg	229	57.7	58.5, 60.5
GATA4-E6	ccggctgtcgtttgtcc	ctctgggactctgcagctgc	269	59.7	62.6, 65.1
GATA4-E7P1	cagcctagacctccaaagc	acaggagagatgcagtgctgc	499	59.1	60.8, 61.4, 62.2
GATA4-E7P2	gacaatctggttaggggaagc	ccagctgcatctttagtagg	470	58.4	59.4, 61.5, 63.3
GATA4-E7P3	gccctgcacccaataacc	cagccctgggacactcc	482	59.8	58.2, 61.2
GATA4-E7P4	agtctggcaagcactcagc	ccagtaggatttggagtgagg	484	59.4	61.2, 62.2
GATA4-E7P5	ctgcacattgctgttctgc	ctacacggcctcaagattcc	384	58.3	56.5, 58.1, 60.6
TBX5-E1P1	ggtattcattgcccagagc	cccagtaaaataaagaggcaacc	478	57.9	53.6, 59.2, 63.5, 64.9
TBX5-E1P2	ccagccaaacgtgacagc	gccaaagtcaagagagaaacc	390	57.8	57.8, 60.4, 62.8
TBX5-E2	tttctctgttctctctgtcc	cagactctgactttgatctctgc	297	60.2	62.8, 66.2
TBX5-E3	gtgttttgggggagttgg	gccacctttctctcacc	243	57	58.9, 60.4
TBX5-E4	gaggctgcctaaataactgg	aactttttgggagaaggtcc	248	56.7	57.7, 60.2
TBX5-E5	ctgtgctgtaactgaagc	gaggacaagaggagacaagc	282	60.3	62.7, 65.1
TBX5-E6	gggagcagggtttatctgg	tgcaaaagaaagagcagacg	280	54.3	54.6, 57.8, 61.1
TBX5-E7	tgcttaattgctcttttgg	ggtgtgctgtgcttacc	294	53.4	56.5, 58.9
TBX5-E8	tctctcacactggttcagc	atactctcaccccctcacc	390	60.3	58.7, 61.3
TBX5-E9P1	ttggccaataactgtctcc	gtggaaacattccctcc	465	54.1	56.3, 60.5, 63.8
TBX5-E9P2	acttctccgctcacttcacc	tttttaaattgtggttcaagc	474	50.3	55.6, 59, 61.3, 63.5
TBX5-E9P3	ggacaagattttcatttcacc	ggtaggtgctttcttagtcaagg	496	53.4	52.8, 56.4, 58, 59.3
TBX5-E9P4	ggaccagtccttatttgg	ttaatcagggagaatattatit	481	49.9	56.9, 58, 59.2
TBX5-E9P5	tgccctatagcttccctcc	ctctggccagctcctatgc	482	60.3	53.1, 54.5, 56.7, 58.4
TBX5-E9P6	tgtgtaagtaagtgattgtagg	aaagagacataatcgcataagg	361	56.9	52.1, 54.2, 57
TBX5-E9P7	aagagaacagggtgaagatgtagg	ttcctgttctcccaattcc	276	54.2	56.6, 58.7

PCR, polymerase chain reaction; dHPLC, denaturing high performance liquid chromatography.

**Figure 1.** Sequence traces showing the nonsynonymous nucleotide changes causing amino acid replacements in the *NKX2.5*, *GATA4*, and *TBX5* proteins. The changes are indicated with arrows.

NP\_004378.1 for the protein. In exon 2, a 541T>C transition (L122P) was discovered in a patient with secundum ASD (Figure 1A). The mutation was transmitted by the unaffected father.

The same patient also harbors a private mutation we reported previously (a 4395C>A transversion resulting in A1443D) in exon 30 of the *MYH6* gene, which was transmitted by the mother.<sup>29</sup> A



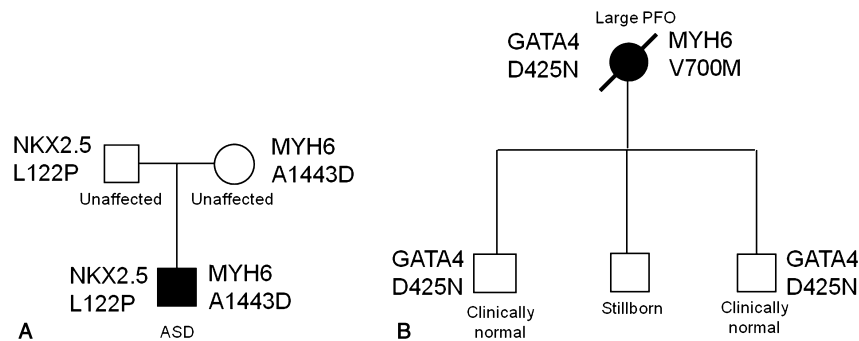
parents are of white descent. The A1443D mutation introduces a negatively charged residue in a position where only noncharged amino acids exist in sarcomeric myosins from mammals, chick, zebrafish, *Xenopus*, and *Caenorhabditis elegans* and is predicted to interfere with the interaction between the two molecules of the myosin dimer or with other sarcomeric proteins.<sup>29</sup> The frequency of the alleles encoding either L122P (*NKX2-5*) or A1443D (*MYH6*) in the CHD and control cohorts is 0.000619. The probability of both private alleles occurring in the same individual by chance, assuming the variants do not have causal relation to the phenotype is estimated to be  $4.82 \times 10^{-7}$ . The most common phenotype resulting from mutations in either *NKX2-5* or *MYH6* is secundum ASD.<sup>15,29,38</sup> As this patient has the same diagnosis, double heterozygosity could at least partially explain the development of the defect in the proband and the incomplete penetrance of the L122P and A1443D mutations in the parents. Incomplete penetrance is a common finding in documented cases of mutations causing Mendelian CHD.<sup>4</sup> In the case of *NKX2-5*, recent data highlight the profound influence of modifier loci in the pathogenesis of single-gene cardiac malformation.<sup>39</sup>

The novel *NKX2-5* G232R mutation occurs in the C-terminal end of the NK2-specific domain (NK2-SD) and introduces a positively charged residue in a position where only noncharged residues exist in every vertebrate *NKX2-5* ortholog sequence available (Figure 4A). The NK2-SD interaction with bone morphogenetic protein (BMP)-dependent mothers against decapentaplegic (SMAD) proteins is required for the binding of the homeodomain with the histone deacetylase (HDAC)/Sin3A complex.<sup>40</sup> This domain is also thought to stabilize the interaction of the tinman

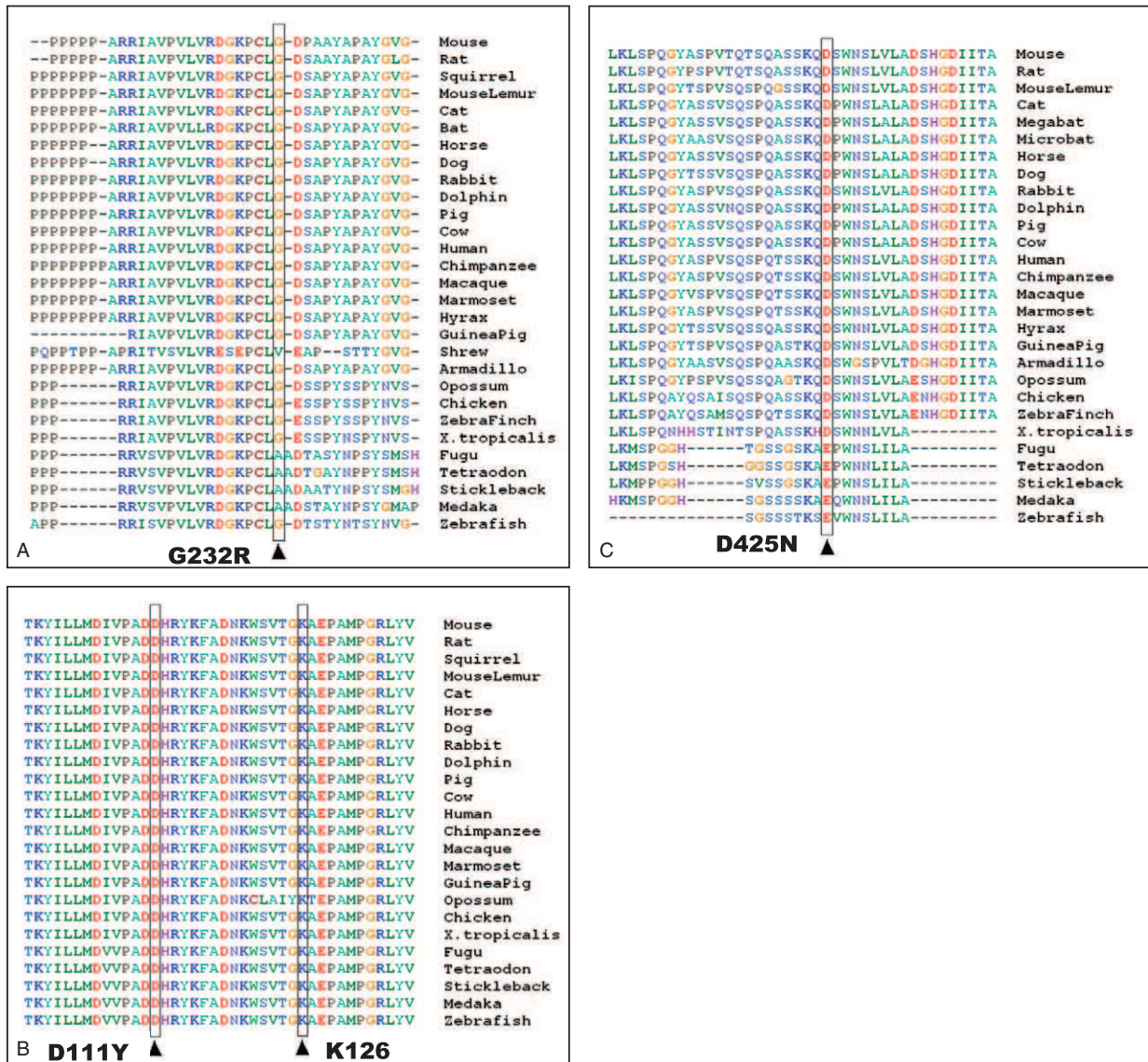
domain to the corepressor Groucho proteins<sup>41</sup> and to modulate, in an intramolecular fashion, the activity of the C-terminal activation domain.<sup>42</sup> These data suggest that the G232R mutation could compromise the transcriptional-repressor activity of *NKX2-5* and its responsiveness to BMP signaling.

The novel D111Y *TBX5* variant occurs within the conserved T-box element of the molecule. A three-dimensional structure model of the human *TBX5* protein (PDB: 2X6U) has been made available recently.<sup>43</sup> This model allows more precise predictions of the consequences of the variant D111Y (Figure 5A). The structural model predicts that the salt bridge present between the K126 and D111 is disrupted when the aspartic acid residue (D) is replaced, as in the case of the D111Y variant, by an uncharged tyrosine (Y) residue (Figure 5B). A model of the human *TBX3* bound to its target sequence (PDB: 1H6F)<sup>44</sup> reveals that the side-chains of both K126 and D111 point to opposite directions during interaction with DNA, canceling the salt bridge (Figure 5C) that suggests that the D111Y variant could have an impact on the conformational change of *TBX5* upon binding to its target promoters. Both residues involved in the salt bridge (D111 and K126) are universally conserved in every *TBX5* vertebrate sequence available (Figure 4B). We suggest that these data warrant the inclusion of the *TBX5* D111Y variant in future case-control studies for sporadic CHD, as this variant could confer susceptibility to the disease.

The D425N mutation of *GATA4* has been reported previously in three patients with secundum ASD, VSD, and tetralogy of Fallot.<sup>34,35</sup> This variation replaces a negatively charged aspartic acid (D) residue for an uncharged asparagine (N) residue in a position where only negatively charged amino acids are found in *GATA4*



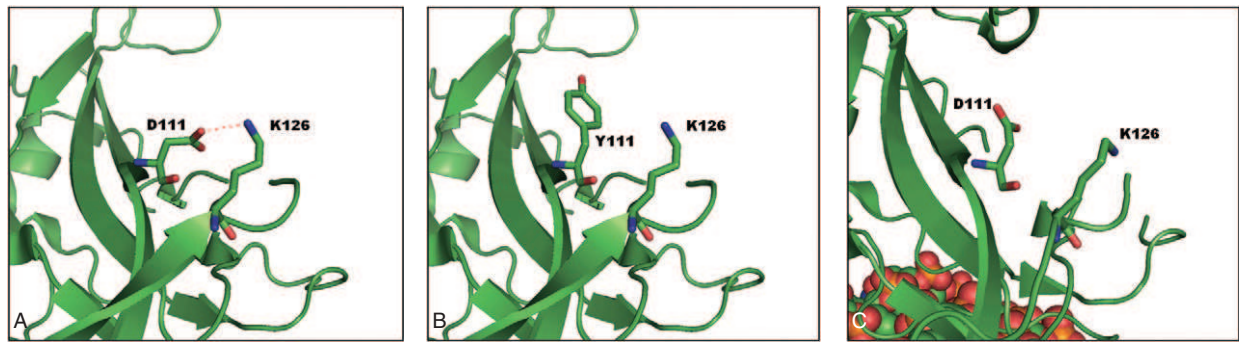
**Figure 3.** Pedigrees of the families CHD where multiple heterozygosity for the *NKX2.5*, *GATA4*, and *MYH6* genes was detected. ASD, atrial septal defect; PFO, patent foramen ovale.



**Figure 4.** Multiple alignment of amino acid sequences of segments of the NKX2-5, GATA4, and TBX5 proteins from every vertebrate species available. (A) The NKX2-5 G232R mutation introduces a positively charged arginine residue in a position where only noncharged amino acids are present in NKX2.5 vertebrate orthologs. (B) The TBX5 D111Y mutation replaces a negatively charged aspartic acid residue with a noncharged tyrosine residue. Both amino acids forming a salt bridge predicted by the human TBX5 tridimensional structure (PDB: 2X6U) are universally conserved among TBX5 vertebrate orthologs. (C) The D425N GATA4 mutation introduces a noncharged residue in a position where only negatively charged residues are present in every vertebrate GATA4 ortholog sequence available.

orthologs in wide range of vertebrate species (Figure 4C). The patient heterozygous for D425N also carries a private mutation in exon 18 of *MYH6*, a 2165G>A transition (V700M) (Figure 3B).<sup>29</sup> This mutation is located within the segment of the *MYH6* gene encoding a part of the SH1 helix that is conserved in every myosin sequence available and is predicted to hinder the movements of the helix at different stages of

the myosin cycle.<sup>29</sup> Two clinically normal sons of the probanda are both heterozygous for the D425N *GATA4* mutation but do not carry the V700M *MYH6* mutation. The patient suffered a stillbirth but no sample from the product was available for analysis. The structure of this pedigree is consistent with complementation of both variants to contribute to the phenotype (Figure 3B).



**Figure 5.** (A) Molecular model of human TBX5 protein free from nucleic acid based on PDB file 2X6U showing the salt bridge between the D111 and the K126 residues. (B) The D111Y change disrupts the salt bridge, as the negatively charged aspartic acid (D) residue is replaced by an uncharged tyrosine residue (Y). (C) Model of the T-box motif bound to nucleic acid based on PDB file 1H6F showing that during interaction with its target promoter, the side-chains of the D111 and K126 residues point to opposite directions, canceling the salt bridge.

## Conclusions

Mendelian nonsyndromic CHD is known to be caused by mutations of genes encoding transcription factors<sup>4</sup> and, notably, muscle proteins expressed in the developing heart or great vessels like ACTC1,<sup>45</sup> MYH6,<sup>29,38</sup> MYH7,<sup>46</sup> and MYH11.<sup>47,48</sup> Here, we describe two novel mutations of *NKX2-5* associated with CHD. Also, we present cases where multiple heterozygosity of variants could contribute, by additive effects, to the development of individual cases of cardiac malformation. Our findings highlight the usefulness of multiple gene analysis in large patient CHD cohorts in order to identify variants whose combined effects may cause the phenotype.

Although array-based genome-wide association studies (GWAS) have been successful in identifying chromosomal segments and variants associated with complex phenotypes, in general, they only explain a small fraction of their heritability.<sup>49</sup> Recent findings suggest that a great proportion of the “missing heritability” in GWAS studies can be masked by incomplete linkage disequilibrium between the causal variants and the single-nucleotide polymorphisms (SNPs) typed by the arrays, as causal variants would tend to have lower minor allele frequencies than the typed SNPs.<sup>50</sup> Thus, we suggest there is a need for further detailed analysis of common, rare, and private variants of genes implicated in Mendelian CHD in association studies aimed at identifying the genetic causes of the more common, sporadic forms of the disease.

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*Conflict of interest:* None.

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## References

- 1 Ferencz C. *Epidemiology of Congenital Heart Disease: The Baltimore-Washington Infant Study 1981–1989*. Mount Kisco: Futura Publishings; 1993.
- 2 Burn J. The aetiology of congenital heart disease. In: Anderson RH, Baker EJ, Macartney FJ, Rigby ML, Shinebourne EA, Tynan M, eds. *Paediatric Cardiology*. London: Churchill Livingstone; 2002: 141–165.
- 3 Nora JJ. Causes of congenital heart diseases: old and new modes, mechanisms, and models. *Am Heart J*. 1993;125:1409–1419.
- 4 Pierpont ME, Basson CT, Benson DW, et al. Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. *Circulation*. 2007;115:3015–3038.
- 5 Garg V, Kathiriyi IS, Barnes R, et al. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature*. 2003;424: 443–447.
- 6 Schott JJ, Benson DW, Basson CT, et al. Congenital heart disease caused by mutations in the transcription factor *NKX2-5*. *Science*. 1998;281:108–111.



- 7 Komuro I, Izumo S. Csx: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci U S A*. 1993;90:8145–8149.
- 8 Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development*. 1993;119:419–431.
- 9 Azpiazu N, Frasch M. Tinman and bagpipe: two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev*. 1993;7:1325–1340.
- 10 Bodmer R. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development*. 1993;118:719–729.
- 11 Harvey RP. NK-2 homeobox genes and heart development. *Dev Biol*. 1996;178:203–216.
- 12 Chen CY, Schwartz RJ. Identification of novel DNA binding targets and regulatory domains of a murine tinman homeodomain factor, nkx-2.5. *J Biol Chem*. 1995;270:15628–15633.
- 13 Stanley EG, Biben C, Elefanty A, et al. Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-Cre allele of the homeobox gene Nkx2-5. *Int J Dev Biol*. 2002;46:431–439.
- 14 Dentice M, Cordeddu V, Rosica A, et al. Missense mutation in the transcription factor NKX2-5: a novel molecular event in the pathogenesis of thyroid dysgenesis. *J Clin Endocrinol Metab*. 2006;91:1428–1433.
- 15 Elliott DA, Kirk EP, Schaft D, Harvey RP. NK-2 class homeodomain proteins: conserved regulators of cardiogenesis. In: Rosenthal N, Harvey RP, eds. *Heart Development and Regeneration*. San Diego, CA: Academic Press; 2010: 569–597.
- 16 Nemer G, Nemer M. GATA4 in heart development and disease. In: Rosenthal N, Harvey RP, eds. *Heart Development and Regeneration*. San Diego, CA: Academic Press; 2010: 599–616.
- 17 Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol*. 1993;13:2235–2246.
- 18 Pehlivan T, Poerber BR, Brueckner M, et al. GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. *Am J Med Genet*. 1999;83:201–206.
- 19 Kennedy SJ, Teebi AS, Adatia I, Teshima I. Inherited duplication, dup(8)(p23.1p23.1) pat, in a father and daughter with congenital heart defects. *Am J Med Genet*. 2001;104:79–80.
- 20 Rajagopal SK, Ma Q, Obler D, et al. Spectrum of heart disease associated with murine and human GATA4 mutation. *J Mol Cell Cardiol*. 2007;43:677–685.
- 21 Basson CT, Bachinsky DR, Lin RC, et al. Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat Genet*. 1997;15:30–35.
- 22 Li QY, Newbury-Ecob RA, Terrett JA, et al. Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat Genet*. 1997;15:21–29.
- 23 McDermott DA, Bressan MC, He J, et al. TBX5 genetic testing validates strict clinical criteria for Holt-Oram syndrome. *Pediatr Res*. 2005;58:981–986.
- 24 Agulnik SI, Garvey N, Hancock S, et al. Evolution of mouse T-box genes by tandem duplication and cluster dispersion. *Genetics*. 1996;144:249–254.
- 25 Fan C, Chen Q, Wang QK. Functional role of transcriptional factor TBX5 in pre-mRNA splicing and Holt-Oram syndrome via association with SC35. *J Biol Chem*. 2009;284:25653–25663.
- 26 Reamon-Buettner SM, Borlak J. TBX5 mutations in non-Holt-Oram syndrome (HOS) malformed hearts. *Hum Mutat*. 2004;24:104.
- 27 Durocher D, Charron F, Warren R, Schwartz RJ, Nemer M. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J*. 1997;16:5687–5696.
- 28 Hiroi Y, Kudoh S, Monzen K, et al. Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet*. 2001;28:276–280.
- 29 Granados-Riveron JT, Ghosh TK, Pope M, et al. Alpha-cardiac myosin heavy chain (MYH6) mutations affecting myofibril formation are associated with congenital heart defects. *Hum Mol Genet*. 2010;19:4007–4016.
- 30 Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res*. 1998;26:1396–1400.
- 31 Tang ZH, Xia L, Chang W, et al. Two novel missense mutations of GATA4 gene in Chinese patients with sporadic congenital heart defects. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2006;23:134–137.
- 32 Schluterman MK, Krysiak AE, Kathiriyai IS, et al. Screening and biochemical analysis of GATA4 sequence variations identified in patients with congenital heart disease. *Am J Med Genet A*. 2007;143A:817–823.
- 33 Butler TL, Esposito G, Blue GM, et al. GATA4 mutations in 357 unrelated patients with congenital heart malformation. *Genet Test Mol Biomarkers*. 2010;14:797–802.
- 34 Tomita-Mitchell A, Maslen CL, Morris CD, Garg V, Goldmuntz E. GATA4 sequence variants in patients with congenital heart disease. *J Med Genet*. 2007;44:779–783.

- 35 Zhang WM, Li XF, Ma ZY, et al. GATA4 and NKX2.5 gene analysis in Chinese Uygur patients with congenital heart disease. *Chin Med J (Engl)*. 2009;122:416–419.
- 36 Kneller DG, Cohen FE, Langridge R. Improvements in protein secondary structure prediction by an enhanced neural network. *J Mol Biol*. 1990; 214:171–182.
- 37 Rey J, Deville J, Chabbert M. Structural determinants stabilizing helical distortions related to proline. *J Struct Biol*. 2010;171:266–276.
- 38 Ching YH, Ghosh TK, Cross SJ, et al. Mutation in myosin heavy chain 6 causes atrial septal defect. *Nat Genet*. 2005;37:423–428.
- 39 Winston JB, Erlich JM, Green CA, et al. Heterogeneity of genetic modifiers ensures normal cardiac development. *Circulation*. 2010;121:1313–1321.
- 40 Kim DW, Lassar AB. Smad-dependent recruitment of a histone deacetylase/Sin3A complex modulates the bone morphogenetic protein-dependent transcriptional repressor activity of Nkx3.2. *Mol Cell Biol*. 2003;23:8704–8717.
- 41 Uhler J, Zhang H, Syu LJ, Mellerick DM. The Nk-2 box of the Drosophila homeodomain protein, Vnd, contributes to its repression activity in a Groucho-dependent manner. *Mech Dev*. 2007; 124:1–10.
- 42 Watada H, Mirmira RG, Kalamaras J, German MS. Intramolecular control of transcriptional activity by the NK2-specific domain in NK-2 homeodomain proteins. *Proc Natl Acad Sci U S A*. 2000;97:9443–9448.
- 43 Stirnimann CU, Ptchelkine D, Grimm C, Muller CW. Structural basis of TBX5-DNA recognition: the T-box domain in its DNA-bound and -unbound form. *J Mol Biol*. 2010;400:71–81.
- 44 Coll M, Seidman JG, Muller CW. Structure of the DNA-bound T-box domain of human TBX3, a transcription factor responsible for ulnar-mammary syndrome. *Structure*. 2002;10:343–356.
- 45 Matsson H, Eason J, Bookwalter CS, et al. Alpha-cardiac actin mutations produce atrial septal defects. *Hum Mol Genet*. 2008;17:256–265.
- 46 Budde BS, Binner P, Waldmuller S, et al. Noncompaction of the ventricular myocardium is associated with a de novo mutation in the beta-myosin heavy chain gene. *PLoS ONE*. 2007;2:e1362.
- 47 Pannu H, Tran-Fadulu V, Papke CL, et al. MYH11 mutations result in a distinct vascular pathology driven by insulin-like growth factor 1 and angiotensin II. *Hum Mol Genet*. 2007;16:2453–2462.
- 48 Zhu L, Vranckx R, Khau Van Kien P, et al. Mutations in myosin heavy chain 11 cause a syndrome associating thoracic aortic aneurysm/aortic dissection and patent ductus arteriosus. *Nat Genet*. 2006; 38:343–349.
- 49 Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. *Nature*. 2009;461:747–753.
- 50 Yang J, Benyamin B, McEvoy BP, et al. Common SNPs explain a large proportion of the heritability for human height. *Nat Genet*. 2010;42:565–569.